ADDRESSING FEASIBILITY OF USING MICRODIALYSIS TO MONITOR ADENOSINE IN ARCTIC GROUND SQUIRREL (*SPERMOPHILUS PARRYII*)

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A

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ABSTRACT

Both hibernation and anapyrexia are important physiological adaptations. In addition, there is a strong relationship between survival rate and hypothermia during metabolic trauma. Evidence shows that adenosine might be one of the mediators, both of hibernation and anapyrexia. Adenosine is an inhibitory neuromodulator that suppresses neuronal activity when energy stores are low. This inhibitory activity makes adenosine a good candidate for neuroprotection via the suppression of neural activity during metabolic trauma.

The main objective of this study was to investigate the feasibility of using microdialysis to monitor extracellular adenosine in the brains of Arctic ground squirrels (AGS), *Spermophilus parryii*, throughout hibernation. To investigate this feasibility, the basal level of adenosine concentration in AGS was collected using an *in vivo* microdialysis method. Samples then were analyzed by high performance liquid chromatography (HPLC). Also, the effects of adding an adenosine transporter inhibitor, S-(p-Nitrobenzyl)-6-thioinosine (NBTI), on extracellular adenosine concentration and body temperature (Tb) of AGS were observed. The results of this study indicate that it is not feasible to observe extracellular adenosine concentrations in AGS during hibernation using these techniques.

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Chapter 1

Introduction

1.1. Adenosine/metabolic suppression

Adenosine is an inhibitory neuromodulator that is linked to energy supply and demand. Adenosine functions as an auto-regulating inhibitory neuromodulator to suppress neuronal activity when energy stores are low. Adenosine inhibits the release of many neurotransmitters, including the excitatory neurotransmitter glutamate. Adenosine also induces the hyperpolarization of neurons in the hippocampus and corpus striatum (Stone, 2002).

There are four adenosine receptors, A_1, A_{2A}, A_{2B} and A_3. Activation of these receptors mediate different effects of adenosine. The A_1 receptor is the most well known and is recognized for its inhibitory effect. Stimulation of the A_{2A} receptor results in excitatory action, whereas both a protective and excitatory effect have been observed with stimulation of the A_3 adenosine receptor (Latini and Pedata, 2001). The consequences of A_{2B} receptor stimulation are not well known.

Activation of the adenosine A_1 receptor is known to produce a neurodepressent effect (Tamura et al., 2005). Adenosine concentrations increase during times of metabolic trauma. For example, although the normal, resting extracellular concentration of adenosine is less than 1 mM, this

concentration can increase up to 30 mM or more under conditions of cerebral ischemia (Stone, 2002). Adenosine's inhibitory activity makes adenosine a good candidate for neuroprotection, because of its suppression of neural activity during brain trauma (Stone, 2002).

Hibernation is characterized by extended bouts of torpor; that is, physical and metabolic dormancy. Torpor is a unique physiological adaptive response that allows animals to survive during prolonged periods of food shortage and extreme weather (Carey et al., 2003). During torpor, an animal lowers its body temperature (Tb) to a few degrees above the ambient temperature, a process that would be harmful for non-hibernating species including humans. In addition, heart, respiratory and metabolic rates are suppressed during hibernation (Carey et al., 2003). Adenosine may play an important role in hibernation due to its ability to suppress neuronal activity.

Anapyrexia (from the Greek words *an*, meaning *an absence of*, and *pyrexia*, meaning *fever)* is a regulated decrease in body temperature (Tb) (Steiner and Branco, 2002). Anapyrexia is a protective and compensatory response that results from hypoxia or other oxygen-limiting environments. Decreased temperature is an important adaptive response that protects vital organs during bouts of metabolic trauma. Anapyrexia results in increased oxygen uptake and decreased oxygen consumption, as well as decreased pulmonary and respiratory hyperactivity, often related to metabolic trauma. For example, a 1°C decrease in brain temperature is adequate to prevent a

decline in brain ATP levels at partial pressures of oxygen around 20 mmHg (Steiner and Branco, 2002). A strong relationship between survival rate and anapyrexia exists during periods of metabolic trauma.

Some evidence suggests that adenosine is a mediator of the anapyrexia that is induced by hypoxia. Many studies show that systemic administration of adenosine antagonists attenuates hypoxia-induced anapyrexia in many species (Steiner and Branco, 2002). In addition, an intracerebroventricular (ICV) injection of an adenosine antagonist has substantially attenuated hypoxia-induced anapyrexia in some animals (Barros and Branco, 2000; Steiner and Branco, 2002). Thus, adenosine is a possible mediator of anapyrexia-induced hypoxia and the central nervous system (CNS) appears to be an important site of its action (Steiner and Branco, 2002).

1.2. Objectives

The primary objective of this research, therefore, was to investigate adenosine concentrations in the Arctic Ground Squirrels (AGS). Our specific objectives were:

1. To develop a sensitive high performance liquid chromatography (HPLC) assay to analyze adenosine concentrations in dialysate that has been collected using microdialysis methods.

2. To measure the basal levels of adenosine concentration in euthermic AGS and then observe the effect of different doses of S-(p-Nitrobenzyl)-6-thioinosine (NBTI) on these levels.

3. To compare the *in vivo* performance of two different probes, the CMA 12 (CMA, Chelmsford, MA) and low dead volume (LDV) probes for measuring adenosine concentrations.

4. To assess the feasibility of using microdialysis to monitor extracellular adenosine in brains of AGS throughout hibernation.

Chapter 2

Results

2.1. Adenosine measurement technique – High performance liquid chromatography (HPLC)

Introduction

Adenosine is an inhibitory neuromodulator that is a good candidate for inducing neuroprotection during brain trauma. With its ability to suppress neuronal activity, adenosine may play an important role in hibernation. In addition, adenosine is known to mediate anapyrexia induced by hypoxia. HPLC is a technique that is commonly used to analyze adenosine in samples collected by *in vivo* microdialysis. Microdialysis is a well-established technique that allows for sampling of low-molecular-weight substances in the extracellular space of the brain. The method depends upon the diffusion of dissolved molecules surrounding the microdialysis probe across a semi-permeable dialysis membrane into the brain (Bourne, 2003).

Several methods for analyzing adenosine are available. Of the various potential methods, HPLC and biosensor techniques are the two main techniques used. Both HPLC and biosensor techniques have good sensitivity. The microelectrode biosensor technique allows for real time *in situ* measurement of adenosine. Moreover, it yields better spatial and temporal resolution than that obtained when using a microdialysis probe (Dale et al.,

2005). However, previously-published microelectrode biosensor techniques are enzyme based. (Dale et al., 2002; Frenguelli et al., 2003; Llaudet et al., 2003). Enzymes work best when operating within a specific temperature range. Above or below their optimum temperature, activity can be decreased or the enzyme can be denatured. The enzymes generally used in biosensor techniques are adenosine deaminase (AD), nucleoside phosphorylase (NP) and xanthine oxidase (XO). Their optimum temperatures are 37°C, 60°C, and 75°C, respectively, which are much higher than cold room temperatures or a hibernating animal's body temperature. In addition, the body temperature of euthermic state of animal is around 37°C which results in much higher enzyme activity. This discrepancy between the temperature of a hibernating animal and its euthermic state could result in significant differences in biosensor enzyme activity levels which, in turn, could significantly alter results. As a consequence of this temperature sensitivity of the enzyme and the wide range of animal body temperature, we felt that the biosensor technique was not suitable for the currently presented research.

Consequently, our goal was to test the published HPLC method used by Porkka-Heiskanen et al. (1997) to establish its limit of detection (sensitivity) and then compare the performance of two different UV detectors.

Methods

Instrumentation

A previously-published method was used to quantify adenosine with HPLC (Porkka-Heiskanen et.al., 1997). The mobile phase consisted of 8mM NaH_2PO_4 in 8% methanol (pH=4). The mobile phase was degassed with a 0.2-µm polypropylene filter device (Whatman INC, Clifton, NJ cat number 6726-5002). A flow rate of 80 µL/min was produced using a Micro LC 100 pump (Pronexus, Stockholm, Sweden). Separation was achieved using a 1 X 100 mm BAS microbore column with C18 packing of 3-µm particle size (MF 8949, BAS, Lafayette, IN). The column was attached to an injector (MF 4161, BAS, Lafayette, IN) with a 20 µL loop (9055-022, Rheodyne, Rohnert Park, CA, USA) and to a UV detector outfitted with a microbore cell kit. A Waters UV 2487 Dual λ absorbance detector (Milford, MA) and a BAS UV-116A UV-VIS detector (Lafayette, IN) were compared. Adenosine was detected at a wavelength of 258 nm. Chromatographic data were recorded using an SP-4290 integrator (Spectra-Physics, San Jose, CA, USA) or Peak Simple 3.29 SRI Inc, State College, PA, USA).

Sensitivity comparison of two different UV detectors

The conditions of the experiment were the same as those described above. Two different UV detectors were used to compare the limits of detection. With each 4 µL injection, adenosine standards at concentrations of

0, 0.05, 0.1, and 1.0 μM were tested. Standard curves were analyzed using each detector for comparison, and the detection limit was calculated based upon a signal-to-noise ratio of 2:1. Each standard concentration was analyzed 5 times to calculate variability.

Confirmation of adenosine in the dialysate sample

The preliminary identification of adenosine was confirmed by spiking samples with known concentrations of the adenosine standard and then treating with adenosine deaminase, which converts adenosine to inosine. The peak heights among microdialysis samples were quantified and compared to the peak heights of the adenosine standards.

Results

As shown in Figure 1, the sensitivity of the Waters UV detector was 10 times greater than that of the BAS detector. The calculated limit of detection based upon a signal to noise ratio of 2:1, was 0.020 pmol with the Waters detector, whereas the BAS detector had a detection limit of 0.25 pmol. The repeated assays of standards showed less than 10% variability at concentrations of 0, 0.05, 0.1, and 1.0 μM for both UV detectors.

Adenosine in the dialysate sample was identified and confirmed using the Waters detector by spiking samples with known concentrations of the adenosine standard and then treating adenosine standard with adenosine

deaminase. As shown in Figures 2 and 3, the adenosine peak increased after spiking with known adenosine standards and decreased after treatment with adenosine deaminase.

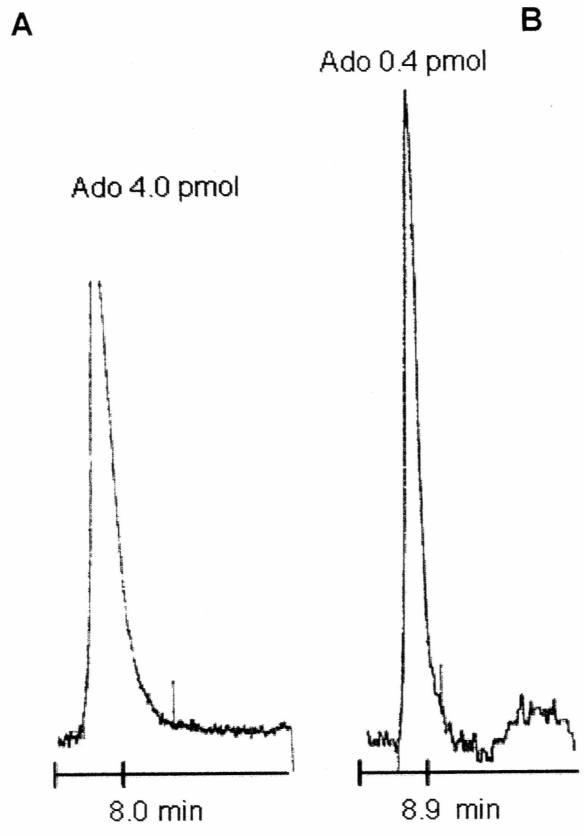

Figure 1. Chromatogram of 4 pmol adenosine standard analyzed with the BAS (A) and Waters UV (B) detectors. (A) Chromatogram of 4 pmol adenosine standard analyzed with the BAS UV detector. 4 µl of an 1.0 µM adenosine standard was injected. The calculated limit of detection based upon a signal to noise ratio of 2:1 was 0.25 pmol with the BAS detector. (b) Chromatogram of 0.4 pmol adenosine standard using the Waters UV detector. 4 µl of 0.1 µM adenosine standard was injected. The calculated limit of detection based upon a signal to noise ratio of 2:1 was 0.020 pmol with the Waters detector.

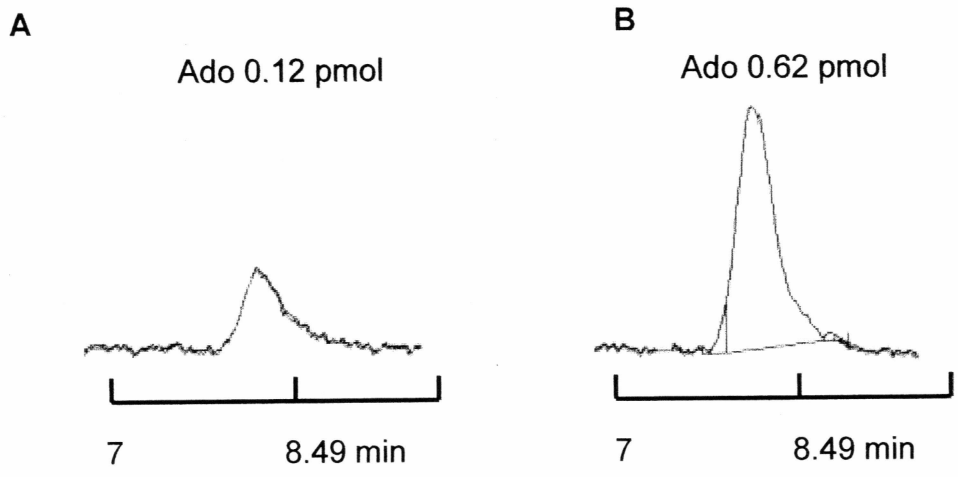

Figure 2. Confirmation of adenosine in the dialysate. Samples of AGS 03-17 RS4, LS3 and RS3 were combined for analysis. (A) Chromatogram of 2 µL of combined sample detected with the Waters UV detector. (B) Chromatogram of a 2 µL combined sample spiked with 2 µL of 0.2 uM adenosine standard (0.4 pmol) using the Waters UV detector.

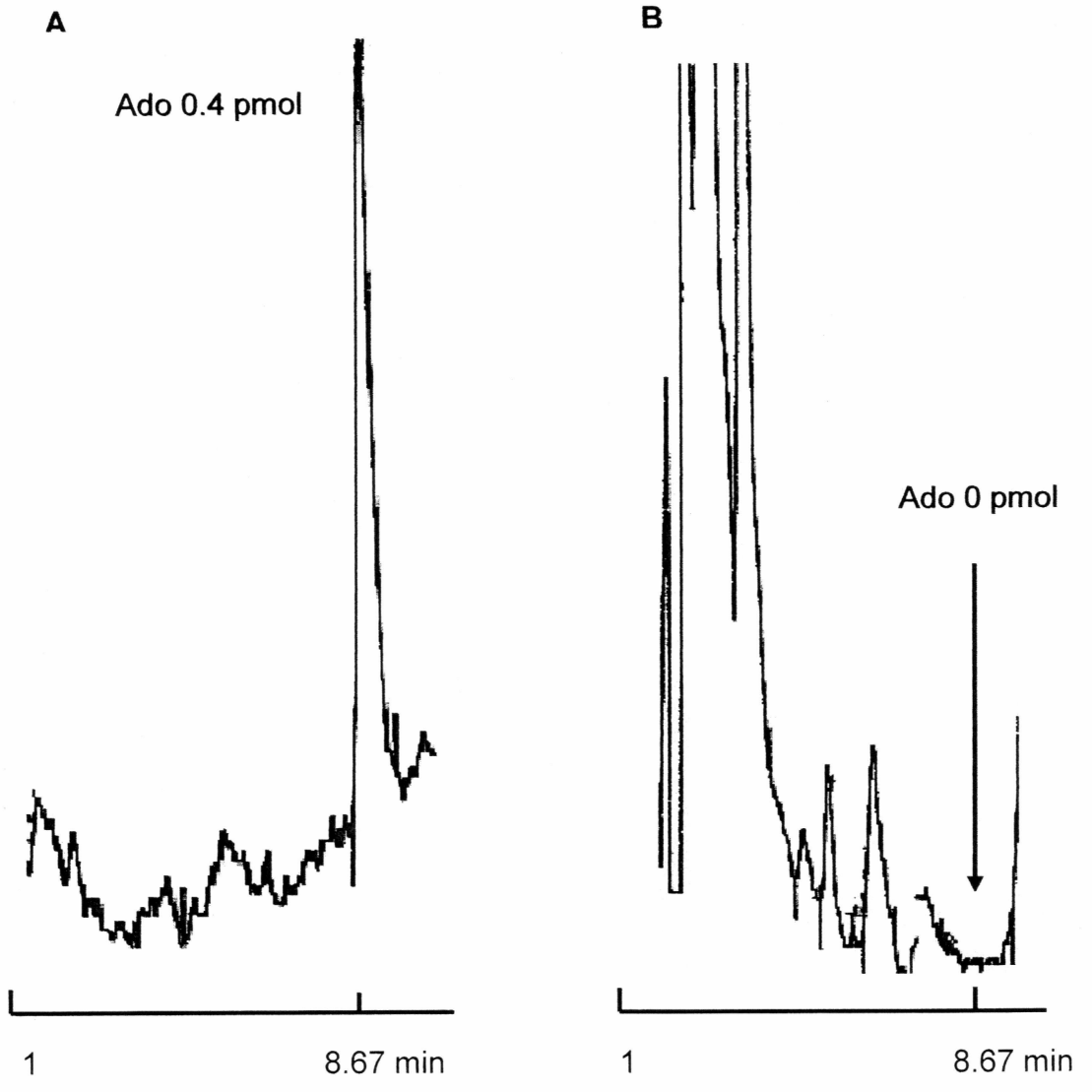

Figure 3. Confirmation of adenosine with adenosine deaminase treatment. (A) Chromatogram of 0.4 pmol of adenosine standard (Waters UV detector). (B) Chromatogram of 0.4 pmol adenosine standard mixed with adenosine deaminase (Waters UV detector).

2.2. Euthermic NBTI experiment – Testing the feasibility of measuring baseline extracellular adenosine concentrations in arctic ground squirrels (AGS)

Introduction

There is a body of evidence that demonstrates a relationship between adenosine and thermoregulation, and provides some insights into adenosine's role in this process. Both systemic injection of adenosine and intracerebro-ventricular (ICV) injection of an adenosine analogue have been shown to decrease body temperature (Tb) (Steiner and Branco, 2002). Another study showed that the activation of A1-receptor by adenosine has a critical role in inducing hypothermia during hibernation (Tamura et al., 2005). The ICV injection of 8-cyclopenthyltheophylline (CPT), an A-1 receptor antagonist, 17 h after the onset of hibernation resulted in interruption of hibernation in hamsters. However, the interruption of hibernation was not observed when the same drug was injected 30 h after the onset of hibernation (Tamura et al., 2005).

The aims of the current study were to test the feasibility of measuring baseline adenosine concentrations in the striatum of the Arctic ground squirrel (AGS) and to observe the NBTI dose response *in vivo*. NBTI is an adenosine transport inhibitor, which blocks adenosine transport and, in some cases, increases extracellular adenosine concentrations. For example, NBTI perfusion in the basal forebrain and thalamus of a cat produced an increase in extracellular adenosine concentrations (Porkka-Heiskanen et al., 1997).

Increasing extracellular adenosine with NBTI perfusion should reveal the relationship between adenosine and body temperature. Therefore, microdialysis samples were collected and analyzed with HPLC to compare adenosine concentrations in the striatum before and during NBTI perfusion. Body temperature was monitored constantly to observe the direct effect of NBTI.

Methods

Surgery

All procedures were approved by the Institutional Animal Care and Use Committee. The surgery was performed under general anesthesia with 1-3 % halothane (Halocarbon Lab, Riveredge, NJ) mixed with 100% medical grade O_2 at a flow rate of 1.5 L/min. All surgical procedures were performed under strictly aseptic conditions. The ground squirrels were fasted for at least 12 hours prior to surgery. After being anesthetized, the AGS were placed in a rat stereotaxic frame (Stoelting, Wooddale, IL) and holes were drilled into the skull to permit placement of guide cannulae. Guide cannulae (CMA, Acton, MA) were sterotaxically positioned above the right and left striatum (AP=13.5 or 14 mm, L=±3.25 mm, D=-4.0 mm) and slowly lowered to a position such that the base of the cannulae were 4 mm below the cortical surface (Osborne et al., 1999). Four anchor stainless steel screws (BAS, West Lafaette, IN) were implanted in the skull and the guide cannulae were secured to the screws with

dental cement. A metal hook used to provide strain relief for microdialysis tubing also was secured in the dental cement. Pre-calibrated telemetry transmitters (model VM-FH, Minimitter, Sunriver, OR) were implanted intraperioneally to monitor core body temperature. An antibiotic (enrofloxacin 5 mg/kg) was given 1 day before surgery and 2 days after surgery by subcutaneous injection. Post-operatively, the ground squirrels were housed individually under a light regime of 12:12h light:dark at 20-22°C for at least 10 days. Next, the ground squirrels were transferred to a cold chamber with an ambient temperature of 2-4°C on a 4:20h light:dark cycle. Hibernation was observed using the shavings-added technique; the ground squirrels were noted to be hibernating if the wood shavings placed on their backs remained overnight.

Microdialysis

Microdialysis procedures were followed as described, in detail, elsewhere (Osborne et al. 1999). However, all components of the microdialysis system were sterilized via heat (autoclave), ethylene oxide (Anprolene, Andersen Products, Haw River, NC) or 0.2 µm filtration (Acrodisc, Pall Corporation, Ann Arbor, MI). The ground squirrels were selected for the microdialysis experiment if a hibernation pattern of at least two episodes occurred, or if an AGS remained euthermic after at least 1 month of cold adaptation. Body temperature was monitored by telemetry using Dataquest

software (Dataquest III, Data Sciences, Minneapolis, MN) at least 1 day before and throughout the microdialysis experiment to verify the hibernation state. On the first day of the experiment, a microdialysis probe with an outer diameter of 0.5 mm (CMA 12, CMA, Chelmsford, MA) was inserted into the left and right striata through the guide cannulae, while animals were hibernating or euthermic. Only euthermic ground squirrels were lightly anesthetized with halothane, induced as previously described, and maintained at 1% for approximately 5 min. The inlet of the microdialysis probes was connected to a perfusion pump (CMA 100, CMA, Chelmsford, MA) with low volume FEP tubing (CMA). Outlets of the microdialysis probes were connected to a microfraction collector (CMA 142, CMA, Chelmsford, MA). A 1.0-ml gas-tight Hamilton glass syringe (VWR, Willard, OH) was used to perfuse artificial cerebral spinal fluid (aCSF) containing 124 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$, 1.4 mM glucose, and 24 mM $NaHCO_3$, adjusted to pH=7.4, Po_2=70-80 mmHg, and Pco_2=30-40 mmHg by bubbling with 95% N_2/ 5% CO_2. After the insertion of the microdialysis probe, aCSF was perfused at 0.6 µL/min for 2 h, collecting samples every 30 min. Then, samples were collected every hour for the next 20 h at a flow rate of 0.6 µL/min. On the second day of the experiment, the perfusion rate was decreased to 0.1 µL/min. Concentrations of 0.1, 1.0, and 10 µM NBTI were prepared and delivered via reverse dialysis to one side of the brain. Each concentration of NBTI was perfused for 2 h at a flow rate of 0.1 µL /min and samples were collected every

30 min. Perfusion fluid was switched to aCSF after 2 h of 10.0 μM NBTI, and samples collected every 60 min overnight. The same procedure was repeated on the third day of the experiment, except that perfusion was performed on the contralateral side of the brain. A detailed drug experiment datasheet is provided in Appendix 1. Collected samples were stored at -80°C and analyzed for adenosine by HPLC coupled with UV detection, two years later.

HPLC determination of adenosine

A previously-published method was used to detect adenosine with HPLC (Porkka-Heiskanen 1997). The mobile phase was 8 mM NaH_2PO_4 in 8% methanol (pH=4) with a flow rate of 80 μL/min produced by a Micro LC 100 pump (Pronexus, Stockholm). Separation was achieved using a BAS microbore column (MF-8949; 1 X 100 mm, with C18 packing of 3-μm particle size), which was attached to the injector (BAS, Lafayette, IN) and to the UV detector outfitted with a microbore cell kit (Waters UV detector, Lafayette, IN). Adenosine was detected at a wavelength of 258 nm. Chromatographic data were recorded on a Spectra-Physics SP4270 integrator or Peak Simple 3.29 SRI Inc, State College, PA, USA).

Results

The striatum was perfused with the adenosine transport inhibitor, NBTI, using a CMA microdialysis probe, so as to study the effect of NBTI upon core body temperature. Animals had an average of 16 h to stabilize after the insertion of the probe and aCSF was perfused during stabilization. After stabilization, NBTI at concentrations of 0.1, 1.0, and 10.0 uM was delivered to the striatum for 2 h intervals. Body temperature did not change significantly as a result of NBTI perfusion (Figure 4).

The collected microdialysis samples were analyzed with HPLC to establish the baseline concentrations of adenosine and the NBTI dose-response effect. However, it was not feasible to measure the baseline adenosine concentrations, or concentrations in samples collected during NBTI perfusion. Of 24 samples analyzed by HPLC, 23 were below the limit of detection. The average limit of detection was 0.028 pmol.

Discussion

NBTI is an adenosine transport inhibitor. NBTI blocks the transport of adenosine and, thereby, increases extracellular adenosine concentrations. An earlier study by Porkka-Heiskanen showed that NBTI perfusion in the basal forebrain and thalamus of a cat increased adenosine concentrations (Porkka-Heiskanen et al., 1997). The relationship between adenosine and body temperature reported in previously published studies suggests that systemic injection of adenosine and intracerebroventricular (ICV) injection of an

adenosine analogue should result in a dose-related temperature decrease in rodents that is similar to that observed with hypoxia-induced anapyrexia (Steiner and Branco 2002). In addition, a recent study attempted by Tamura et al. (2005) shows that the activation of the A1-receptor by adenosine is important in the generation of hypothermia. However, in the present study, NBTI failed to induce a dose-related decrease in temperature during perfusion of the striatum. Since NBTI failed to induce anapyrexia in these experiments, it is likely that the striatum is not the site of adenosine-induced anapyrexia.

The thermoregulatory set point is located in the pre-optic region of the anterior hypothalamus (PO/AH), and this anatomic site may be the anatomic site of interest for future research (Steiner and Branco, 2002).

It was not feasible to detect adenosine in the baseline samples nor in samples collected after stabilization at a flow rate of 0.1 µL/min. Nor was it possible to detect adenosine in dialysates during NBTI perfusion when analyzed with HPLC, during which the average HPLC limit of detection was 0.028 pmol. Due to instrumentation problems, such as establishing the limit of detection and optimizing the HPLC instruments, samples were kept at -80°C for 2-4 years before being analyzed. The concentration of adenosine in aqueous solution kept at low temperatures over a period of 6 months is very stable (Proot et al., 1998). Although aqueous adenosine is very stable, stability of adenosine in the dialysate might be different, especially when stored for 2-4 years. In addition, a microdialysis sample contains many other

unknown substances as well and degradation of these may contribute to interfering peaks that limit ability to detect adenosine.

During collection at a slow perfusion rate of 0.1 µL/min, samples were exposed to the cold room temperature for long periods of time. With a 1 m long outlet tubing and a slow pump rate, the theoretical duration that perfusate remained in the tubing was 2 h. In addition, the sample collection interval was 30 min per sample. The samples were collected using a microfraction collector. When 7-10 samples were collected with this microfraction collector, samples were sealed, labeled, and kept at -80°C freezer. Therefore, samples were exposed to the 2°C for at least 2.5 h and up to a maximum of 12.5 h. While aqueous adenosine exposed to 2°C should not alter adenosine concentrations, the stability of adenosine and other unknown molecules in the dialysate sample is not known. This long exposure may have caused sample degradation. Decreasing the dead volume by using a shorter outlet tubing (< 6 in) and a smaller dead volume probe may be desirable for future experiments (Khan and Shuaib, 2001). Furthermore, it would be desirable, in future experiments, to analyze samples when the samples are fresh, and to test the stability of adenosine in dialysate samples. The lack of detection may be due to possible sample degradation of adenosine and other unknown substances during sample collection and extended storage.

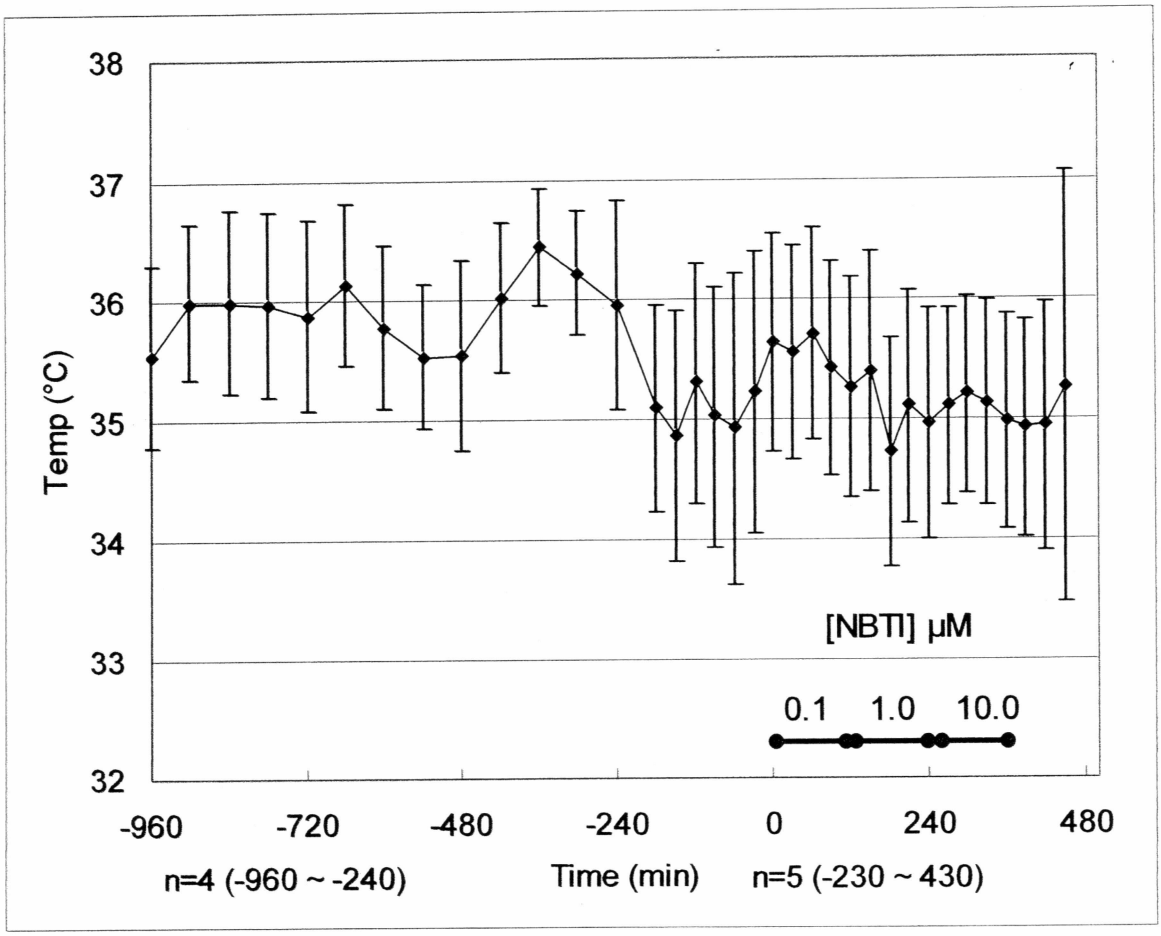

Figure 4. Effect of NBTI on core body temperature. The time, 0 to 360 min, indicates the duration of NBTI perfusion to striatum. NBTI was perfused at 0.1, 1.0, and 10.0 µM concentrations. The core body temperature of AGS is expressed as mean ± SEM. Microdialysis perfusion of NBTI to striatum did not affect core body temperature. N= 4 (Time=-906 to -240); n= (Time=-230 to 430)

2.3. NBTI dose effects on acute non-survival experiment – comparison of CMA and low dead volume (LDV) probes

Introduction

Evidence suggests that the thermoregulatory set point is controlled within the pre-optic region of the anterior hypothalamus (PO/AH) (Steiner and Branco 2002). The main purpose of this study, therefore, was to establish the coordinates of the PO/AH and to observe the effects of NBTI on adenosine concentration. In addition, the *in vivo* performance of two probes was compared: CMA 12 probes and low dead volume (LDV) probes. The advantage of using a CMA probe is that it can be purchased commercially. The advantage of the LDV probe is its smaller size which results in greater temporal resolution and less tissue damage. The specifications for each probe are shown in Table 1. The LDV probe has an outer diameter that is less than half that of the CMA probe. Insertion of the microdialysis probes into the brain produces a good model of penetrating brain injury (Zhou et al., 2001). It follows, then, that these two different-sized probes would produce different degrees of injury.

The effects of NBTI on extracellular adenosine concentration may depend upon this degree of injury. The 5'-nucleotidase pathway becomes the main source of intracellular adenosine under conditions of metabolic trauma, and an efflux of adenosine can be observed. The acute implantation of a probe during the experimental procedure should result in up-regulation of the

5'-nucleotidase pathway. Therefore, the results may vary depending upon the degree of tissue injury.

Methods

Surgery

All procedures were approved by the Institutional Animal Care and Use Committee. The procedure was acute non-survival surgery. Acute implantation of a microdialysis probe was done during the surgery (Drew et al., 1989). The surgery was performed under general anesthesia with 5% isoflorane mixed with 95% medical grade O_2 at a flow rate of 1.5 L/min. All surgical procedures were performed under strictly aseptic conditions. Details of the surgical procedure are described in Drew et al. (1989), except that all components were sterilized by autoclaving, ethylene oxide or 0.2 um filtration as described by Zhou et al. (2001).

Microdialysis

The microdialysis procedures utilized were the same as those described previously, except that perfusion of NBTI was done to both sides of the brain at the same time. After insertion of the microdialysis probe, artificial cerebrospinal (aCSF) was perfused at a rate of 0.6 µL/min for 90 min, with samples collected at 10-minute intervals. Then, the perfusion fluid was switched to 10.0 µM NBTI and for 1 h, and then again switched to aCSF for a 1

h perfusion. Collected samples were stored at -80°C and analyzed for adenosine within 2 weeks.

HPLC determination of adenosine

The same HPLC method was used as previously described.

Statistics

Dialysate concentrations of adenosine in AGS are expressed as means ± SEM. Data collected *in vivo* were analyzed using repeated-measures analysis of variance (ANOVA) (SAS for windows, version 8, SAS Institute INC., Cary, NC). All sample analyses were performed on raw data. The criterion for statistical significance was $p<0.05$.

Results

We compared the *in vivo* performance of the CMA and LDV probes following local perfusion of the hypothalamus with the adenosine transport inhibitor, NBTI.

When using a CMA probe during onset of the 10.0 μM NBTI to the brain, a significant decrease of extracellular adenosine was observed (Figure 5). Extracellular adenosine concentrations decreased during NBTI perfusion. In addition, when the NBTI perfusion was switched to aCSF, the adenosine concentration increased slightly. However, no significant differences in

adenosine concentration were observed during local perfusion of 10.0 μM NBTI using the LDV probe (Figure 6).

Figure 7 shows a comparison of the CMA and LDV probes with respect to percentage change in extracellular adenosine concentrations from baseline to post-NBTI perfusion. The mean of three samples before onset of NBTI perfusion was used to calculate the percentage change from baseline. Adenosine concentration stabilized faster with the LDV probe than with the CMA probe.

Discussion

Adenosine is formed both in intracellular and extracellular spaces. Intracellular adenosine is produced by two major pathways: via the 5'-nucleotidase pathway and by cleavage of S-adenosylhomocysteine (SAH). Extracellular AMP is converted to adenosine by ecto-5'-nucleotidase. The extracellular concentration of adenosine results from an equilibrium between intracellular and extracellular pools of adenosine through bidirectional nucleotide transport that is concentration gradient dependent (Siegal et al., 1999). The concentrations of extracellular adenosine increase when oxygen demand is higher than oxygen supply (Siegal et al., 1999). The resting extracellular concentration of adenosine is 1 mM or less, and it increases to 30 mM under conditions of ischemia or metabolic stress (Stone, 2002). In

addition, most adenosine originates from the intracellular 5'-nucleotidase pathway during these conditions (Siegal et al., 1999).

During acute trauma, most adenosine is derived from the 5'-nucleotidase pathway. With an equilibrative transporter, adenosine will efflux to the extracellular space to balance the adenosine concentration, resulting in an increase in the concentration of extracellular adenosine. However, during our experiment, the equilibrative transporter was blocked with NBTI, which led to blockage of adenosine efflux and a decrease in extracellular adenosine. Using the CMA microdialysis probe resulted in a larger degree of injury than using the LDV microdialysis probe, because the CMA microdialysis probe is larger. This large degree of injury that results from using the CMA microdialysis probe likely was responsible for up-regulation of 5'-nucleotidase which, in turn, resulted in an efflux of adenosine. Moreover, blocking the transporter with NBTI decreased the extracellular adenosine concentration, as shown in Results.

No differences in the concentration of extracellular adenosine were detected using with the LDV microdialysis probe. This might have been due to the smaller degree of injury caused by its smaller size. Since the damage is much less severe, the intracellular 5'-nucleotidase pathway might not be the main source of adenosine production. Therefore, blocking the nucleoside transporter may not affect extracellular adenosine levels.

Our results suggest that extracellular adenosine concentrations stabilize faster with the LDV probe than with the CMA probe. The insertion of a microdialysis probe into the brain is a good brain injury model, comparable to an acute brain trauma. The faster stabilization with the LDV probe was expected, since the outer diameter of the LDV probe is one-half that of the CMA probe and the membrane length is one-fourth shorter. Thus, less damage to the brain occurred during insertion of the smaller probe and this likely led to faster stabilization. This interpretation is consistent with the data, whereby NBTI perfusion using the LDV probe resulted in no significant differences in extracellular adenosine concentrations.

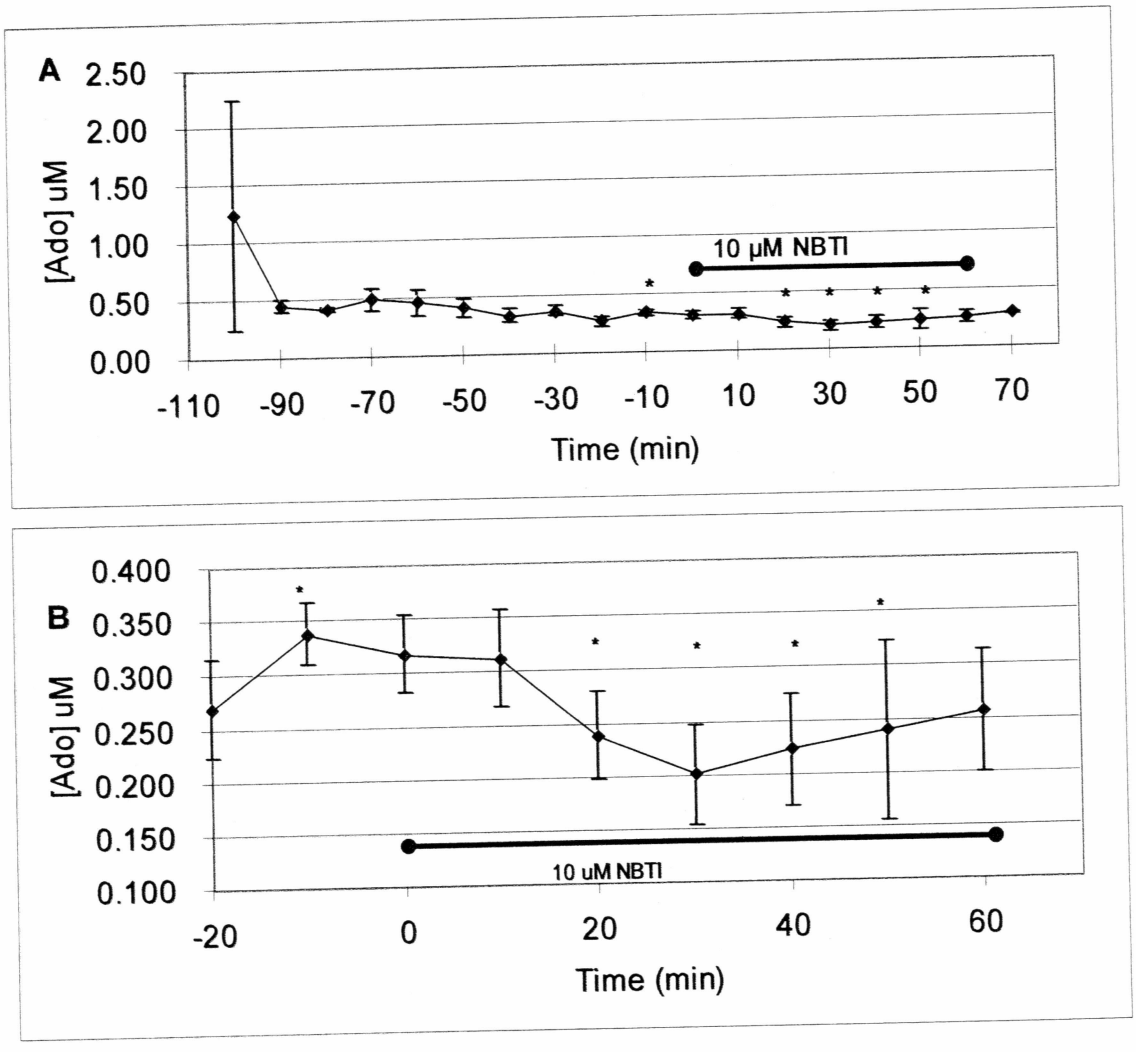

Figure 5. Effects of local perfusion of the adenosine transport inhibitor, NBTI, (10.0 µM) using a CMA probe. Time 0 indicates the onset of 10 µM NBTI perfusion to the hypothalamus. B shows results from -20 to 60 min in A on a smaller scale. Microdialysis perfusion of 10 µM NBTI decreased adenosine concentrations in the hypothalamus. Repeated measures ANOVA comparing treatments revealed a significant difference in adenosine concentration during NBTI perfusion. * $p<0.05$ vs. -10 min; N=3

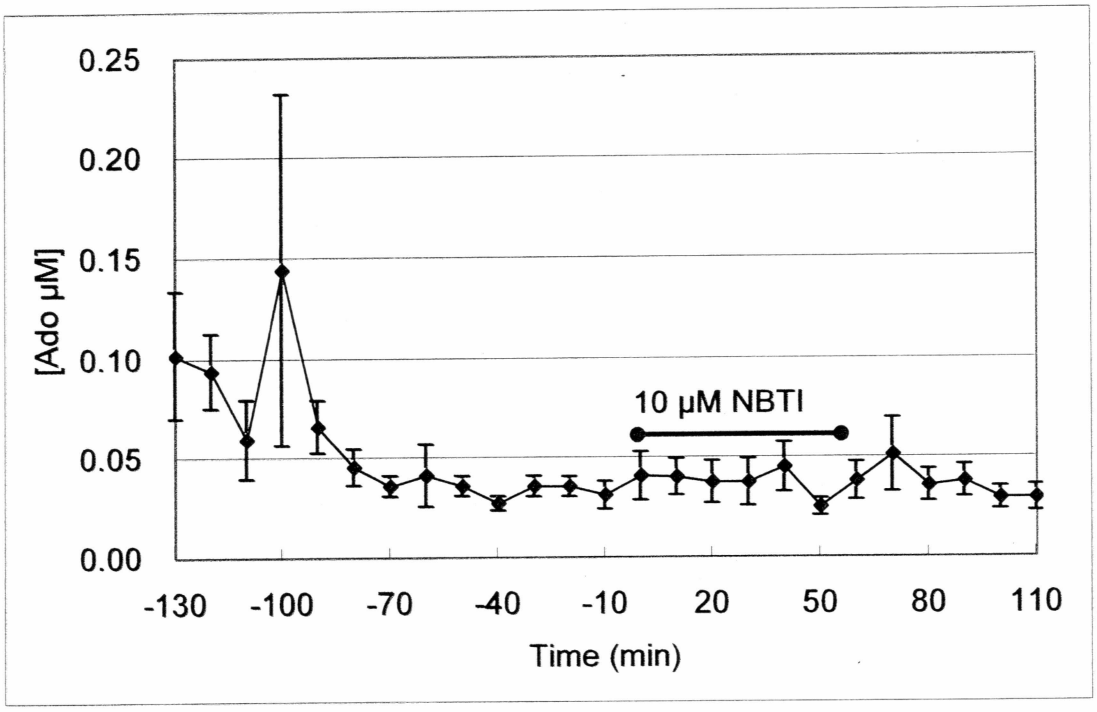

Figure 6. Effects of local perfusion with the adenosine transport inhibitor, NBTI, (10.0 µM) using a LDV probe. Time 0 indicates the onset of 10 µM NBTI perfusion to the hypothalamus. Microdialysis perfusion of NBTI did not affect adenosine concentrations in the hypothalamus. Repeated measures ANOVA comparing treatments showed no significant differences in adenosine concentrations during NBTI perfusion. N=5

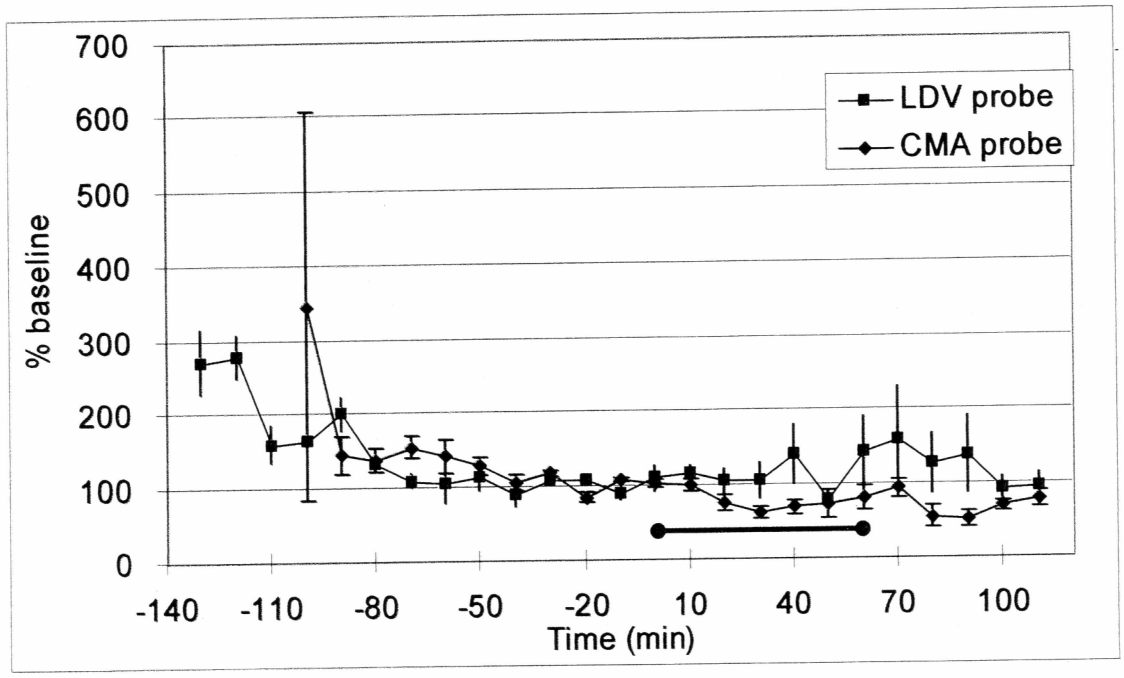

Figure 7. Percent change versus baseline comparison of 10.0 μM NBTI perfusion using the CMA and LDV probes. Time 0 indicates the onset of 10.0 μM NBTI perfusion to the hypothalamus. Use of the LDV probe resulted in faster stabilization of adenosine than with the CMA probe.

Table 1. Specification comparison of two probes: CMA 12 and LDV probes.

	OD (mm)	Membrane Length (mm)	Internal Volume (μL)
CMA 12 probe	0.5	4	3
LDV probe	0.216	1	0.025

Chapter 3

Conclusions

Objective 1 was to develop a sensitive high performance liquid chromatography (HPLC) assay to analyze adenosine concentrations in dialysate that has been collected using microdialysis methods. The limit of detection of 0.02 pmol/4 µL was established by using and adjusting the published HPLC method with our available instruments.

Our second objective was to measure the basal levels of adenosine concentrations in euthermic AGS and then to observe the dose-response effect of NBTI on body temperature and adenosine concentrations. The basal levels of adenosine concentrations in euthermic AGS were not detected and NBTI dosage did not effect body temperature in AGS. However, the extracellular adenosine concentrations within the acute experiment using the CMA probe did demonstrate significant differences.

A comparison of the *in vivo* performance of two different probes, the CMA and low dead volume (LDV) probes, showed that the LDV probe resulted in lesser degree of injury than the CMA probe.

Objective 4 was to assess the feasibility of using microdialysis to monitor extracellular adenosine in brains of AGS throughout hibernation. The results of this study indicate that it is not feasible to observe extracellular adenosine concentration in AGS during hibernation with these techniques. However further modification of the experiments, for example, by increasing

the limit of detection and testing fresh samples, might enable us to achieve this goal in the future.

References

Barros RC, Branco LG (2000) Role of central adenosine in the respiratory and thermoregulatory responses to hypoxia. Neuroreport 11:193-197.

Bourne JA (2003) Intracerebral microdialysis: 30 years as a tool for the neuroscientist. Clinical and Experimental Pharmacology and Physiology 30:16-24.

Carey HV, Andrews MT, Martin SL (2003) Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev 83:1153-1181.

Dale N, Gourine AV, Llaudet E, Bulmer D, Thomas T, Spyer KM (2002) Rapid adenosine release in the nucleus *tractus solitarii* during defence response in rats: real-time measurement *in vivo*. J Physiol 544:149-160.

Dale N, Hatz S, Tian F, Llaudet E (2005) Listening to the brain: microelectrode biosensors for neurochemicals. Trends Biotechnol 23:420-428.

Drew KL, O'Connor WT, Kehr J, Ungerstedt U (1989) Characterization of gamma-aminobutyric acid and dopamine overflow following acute implantation of a microdialysis probe. Life Sci 45:1307-1317.

Frenguelli BG, Llaudet E, Dale N (2003) High-resolution real-time recording with microelectrode biosensors reveals novel aspects of adenosine release during hypoxia in rat hippocampal slices. J Neurochem 86:1506-1515.

Khan SH, Shuaib A (2001) The technique of intracerebral microdialysis. Methods 23:3-9.

Latini S, Pedata F (2001) Adenosine in the central nervous system: release mechanisms and extracellular concentrations. J Neurochem 79:463-484.

Llaudet E, Botting NP, Crayston JA, Dale N (2003) A three-enzyme microelectrode sensor for detecting purine release from central nervous system. Biosens Bioelectron 18:43-52.

Osborne PG, Hu Y, Covey DN, Barnes BN, Katz Z, Drew KL (1999) Determination of striatal extracellular gamma-aminobutyric acid in non-hibernating and hibernating arctic ground squirrels using quantitative microdialysis. Brain Res 839:1-6.

Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW (1997) Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. Science 276:1265-1268.

Proot P, Van Schepdael A, Raymakers AA, Hoogmartens J (1998) Stability of adenosine in infusion. J Pharm Biomed Anal 17:415-418.

Siegal GJ, Agranoff BW, Albers RW, Fisher SK, Uhler MD, editors (1999) Basic Neurochemistry, Molecular, Cellular, and Medical Aspects, 6th ed Edition. Philadelphia: Lippincott, Williams & Wilkins.

Steiner AA, Branco LG (2002) Hypoxia-induced anapyrexia: implications and putative mediators. Annu Rev Physiol 64:263-288.

Stone TW (2002) Purines and neuroprotection. Adv Exp Med Biol 513:249-280.

Tamura Y, Shintani M, Nakamura A, Monden M, Shiomi H (2005) Phase-specific central regulatory systems of hibernation in Syrian hamsters. Brain Res 1045:88-96.

Zhou F, Zhu X, Castellani RJ, Stimmelmayr R, Perry G, Smith MA, Drew KL (2001) Hibernation, a model of neuroprotection. Am J Pathol 158:2145-2151.

Appendix
Euthermic experiment data sheet

Day 2
Experiment: Microdialysis *in vivo* Date:_____

Animal Species:_____AGS___ DQ species: Rat

Animal ID# Perfusion Fluid:_____ACSF___

Brain Side drug delivery on : Left / Right

Flow rate:0.1 ul/min ***Collect the overnight Samples, label them and put in -80 freezer**

Sample #	drug/Vial	Hour	DQ Note #	Collec.Time	Temp.	*Drug Delivery Schedule*
26		24	1	10:16-10:46 am		
27		25	2	10:46-11:16 am		
28		26	3	11:16-11:46 am		
29		27	4	11:46-12:16 pm		
30		27.5	5	12:16-12:46 pm		**12:16 am change collection time to 30min**
31		28	6	12:46-01:16 pm		Deliver 0.1 uM NBTI
32		28.5	7	01:16-01:46 pm		
33		29	8	01:46-02:16 pm		
34	0.1 NBTI	29.5	9	02:16-02:46 pm		**2:16 pm deliver 1.0 uM NBTI**
35	0.1 NBTI	30	10	02:46-03:16 pm		
36	0.1 NBTI	30.5	11	03:16-03:46 pm		
37	0.1 NBTI	31	12	03:46-04:16 pm		
38	1.0 NBTI	31.5	13	04:16-04:46 pm		**4:16 pm Switch from 1.0 to 10 uM NBTI**
39	1.0 NBTI	32	14	04:46-05:16 pm		
40	1.0 NBTI	32.5	15	05:16-05:46 pm		
41	1.0 NBTI	33	16	05:46-06:16 pm		
42	10 NBTI	33.5	17	06:16-06:46 pm		**6:16 pm**-switch from 10uM to aCSF
43	10 NBTI	34	18	06:46-07:16 pm		**Collect the Samples, Label them and put in -80**
44	10 NBTI	34.5	19	07:16-07:46 pm		
45	10 NBTI	35	20	07:46-08:16 pm		
46		35.5	21	08:16-08:46 pm		
47		36	22	08:46-09:16 pm		
48		36.5	23	09:16-09:46 pm		
49		37	24	09:46-10:16 pm		
50		37.5	25	10:16-10:46 pm		
51		38.5	26	10:46-11:46 pm		**10:46 pm change collection time back to 60min.**
52		39.5	27	11:46-12:46 am		
53		40.5	28	12:46-01:46 am		
54		41.5	29	01:46-02:46 am		
55		42.5	30	02:46-03:46 am		

Glossary of Acronyms

aCSF – artificial cerebral spinal fluid

AD – adenosine deaminase

Ado – adenosine

AGS – Arctic ground squirrel

CNS – central nervous system

CPT – 8-cyclopenthyltheophylline

FEP – Fluorinated Ethylene Propylene

HPLC – high performance liquid chromatography

ICV – intracerebroventricular

LDV – low dead volume

NBTI – S-(p-Nitrobenzyl)-6-thioinosine

NP – nucleoside phosphorylase

PO/AH – pre-optic region of the anterior hypothalamus

SAH – S-adenosylhomocysteine

Tb – body temperature

XO – xanthene oxidase